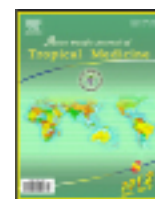


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Molecular detection of *Leishmania* isolated from Cutaneous leishmaniasis patients in Jask County, Hormozgan Province, Southern Iran, 2008

Korosh Azizi¹, Aboozar Soltani^{2*}, Hamzeh Alipour¹¹Department of Medical Entomology, Schools of Health and Nutrition, Shiraz University of Medical Sciences, Shiraz, Iran²Department of Medical Entomology and Vector Control, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

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ABSTRACT

Objective: To investigate on patients leishmanial infections in Jask County. **Methods:** Impression smears were prepared from patients in 2008, all, were checked for leishmanial infection by microscopy and molecular assays. Whole DNA was extracted using Proteinase K and Phenol/Chloroform/Isoamyl alcohol method. The variable segment on minicircles of kinetoplast DNA was amplified via a Nested-PCR technique using species-specific primers (LIN R4–LIN 17–Lin 19). **Results:** A total of 40 smears were prepared from 20 patients, from which, eight samples (40%) were positive for leishman body by microscopic method, while, 18 samples (90%) were positive, molecularly. The parasite was identified as *Leishmania major* (*L. major*). **Conclusions:** Zoonotic or Rural cutaneous leishmaniasis is endemic in Jask County whose pathogen is *L. major*. Molecular assays using specific primers are very accurate and more sensitive and specific than microscopy which is time consuming and needs master microscopists.

1. Introduction

Leishmaniasis are diseases caused by protozoan parasites transmitted via bites of infected sand flies. The differing manifestations of the disease arise from infections with different species of *Leishmania*. Leishmaniasis is still one of the world's most important neglected diseases, mainly affecting the poor caste of the developing communities[1]. 350 million people in 88 countries of developing countries of four continents are considered to be at the risk of infection, two million new cases occur yearly, of which an estimated 500 000 are visceral and 1.5 million cases are cutaneous (90% of them occurring in Afghanistan, Algeria, Brazil, the Islamic Republic of Iran, Peru, Saudi Arabia, Sudan and the Syrian Arab Republic)[2]. Since 1993, the distribution of leishmaniasis has expanded, and there has been a sharp increase in the number of cases recorded[3].

The cutaneous form is more common. It usually causes ulcers on the face, arms and legs. Although the ulcers heal spontaneously, they cause serious disability and

leave severe and permanently disfiguring scars. The cutaneous form may produce up to 200 lesions and lead to disability. The patient is left permanently scarred, and so may become socially stigmatized[4]. Cutaneous leishmaniasis (CL) continues to be an increasing public-health problem in Iran. This form can be seen in zoonotic (ZCL) or anthroponotic (ACL) forms. It is endemic in half of the 30 Iranian provinces. ZCL is widespread in the central, southern and eastern provinces of Iran, mostly caused by *Leishmania major* (*L. major*).

In the recent years several new endemic foci have been reported, indicating the potential spread of disease in the country. During 2001–2008, the number of CL cases has been progressively increased from 11 505 to 26 824 in the country[5]. Jask County which is located in south eastern part of Iran has recently been introduced as the most important endemic focus of cutaneous leishmaniasis in Hormozgan province (245 and 195 cases in 2007 and 2008, respectively) [6].

PCR-based techniques now days are routinely used for detection of *Leishmania* infections, provides a rapid, sensitive, and specific alternative for previous traditional techniques. Moreover, diagnosis of *Leishmania* infection and species identification is done, simultaneously[7–10]. This study was designed to investigate on human infections

*Corresponding author: Aboozar Soltani. Department of Medical Entomology & Vector Control, School of Public Health & National Institute of Health Research, Tehran University of Medical Sciences, P. O. Box: 6446 Tehran 14155, Iran.

E-mail: soltani_a@razi.tums.ac.ir; abu2sol@yahoo.com

in this new-emerged focus because any effective control programmes should be based on the accurate baseline information about disease form and pathogen species.

2. Materials and methods

2.1. Study area

Hormozgan Province covers an area of 71 139.62 km². It is located in south of Iran and north of the Persian Gulf. The Jask County (≈ 154 km²) is a wide aeolic plain on the northern coastline of Oman Sea (25° 38' N, 57° 46' E, at an altitude of 4.8 m a.s.l.) (Figure 1). A roughly 30 km littoral plain belt leads to hilly regions. It is characterized by long dry summers and has a hot humid climate over most of its arid and semi-arid regions with sandy hills. The average of total annual rainfall, mean relative humidity and the mean daily temperature were 136.4 mm, 69.5% and 27.3 °C during 2004–2008, respectively. (www.weather.ir).



Figure 1. Location of sampling zones

2.2. Sampling

The present study was descriptive; cross sectional has been done in 2008. Sampling was achieved from 20 patients (about 10 percent of patients) with acute cutaneous ulcers according to the data of Jask health center. Seven villages containing Lyrdafe, Soorak, Negar, Zikdafi, Koohert, Shemshi and Kangan which had been previously reported as infected areas were considered as sampling zones. Impression smears were prepared on two microscopic slides for each patient. These slides were investigated for leishman body (leptomonad) after fixation by Methanol and Giemsa staining.

2.3. DNA extraction

All microscopically positive and negative slides were then checked for *Leishmania* kDNA by molecular assays. Each dry smear was scraped off the slide with a sterile scalpel and the scrapings transferred to a 1.5 mL reaction tube^[11].

The smear scrapings were added to 150 μ L lysis buffer [50 mM Tris-HCl (pH 7.6), 1 mM EDTA (pH 8.0), 1% Tween 20, and 8.5 M proteinase K solution (19 mg/mL)] and incubated for 2 h at 55 °C. The lysate was then extracted twice with

phenol:chloroform before the DNA was precipitated with ethanol, resuspended in 100 μ L double-distilled water and stored at 4 °C^[10, 12].

2.4. Detection and identification of *Leishmania* species

The variable segment on minicircles of kinetoplast DNA (kDNA) was amplified in a two-rounds, Nested-PCR technique using species-specific primers (LINR4: 5'-GGGGTTGGTGTAATAAGGG-3' as the forward; LIN17: 5'-TTTGAACGGGATTTCTG-3' as the first-step reverse and LIN19: 5'-CAGAACGCCCCCTACCCG-3' as the second-step reverse). The Nested-PCR was carried out in two steps, both in the same tube, as described by Aransay *et al*^[13]. The first-round reaction mixture contained 250 μ M deoxynucleoside triphosphate (dNTP), 1.5 μ M MgCl₂, 1 U Taq polymerase, 1 μ M LINR4, 1 μ M LIN17 and 5 μ L DNA extract in 1 \times PCR buffer in a final volume of 25 μ L. This mixture was incubated in a CG1-96 thermocycler set to run for 5 min at 94 °C, followed by 30 cycles each of 30 s at 95 °C, 1 min at 52 °C, 1 min at 72 °C and a final extension at 72 °C for 10 min and kept at 4 °C. The first-round product (2 μ L of a 4:1 dilution in ddH₂O) was used as template for the second round, in a total volume of 20 μ L and under similar conditions to those for the first round but using LINR4 and LIN19 as the primers in 33 cycles^[10, 14, 15].

This PCR was able to identify promastigote infection of sand flies or amastigote infection in clinical samples and shows specific bands for each *Leishmania* species. The band size was 560 and 720 bp for *L. major* and *Leishmania tropica* (*L. tropica*), respectively^[13, 15]. *Leishmania* species identification was done comparing with standard strains after electrophoresis of PCR products. In order to comparison and confirmation of identified *Leishmania* species, three strains (*Leishmania infantum* (*L. infantum*): MCAN/IR/96/Lon 49, *L. tropica*: MHOM/IR/89/ARD 2, *L. major*: MHOM/IR/54/LV 39) which were kept in the Leishmaniasis laboratory of Parasitology Department at Tehran and Shiraz university, were used as the standard strains.

3. Results

A total of 40 smears were prepared from 20 patients. Smears were examined by two diagnostic techniques, direct (microscopic) examination and a species-specific Nested-PCR. Eight out of 20 samples (40%) were positive for leishman body by microscopic examination and 18 samples (90%) were shown to be positive for kDNA by molecular assay.

Leishman body of *L. major* observed in eight prepared smears (Figure 2). Infective parasite was identified as *L. major* using the nested PCR method. The size of all amplified products was about 560 bp equal to the band size of *L. major* standard strain (Figure 3).

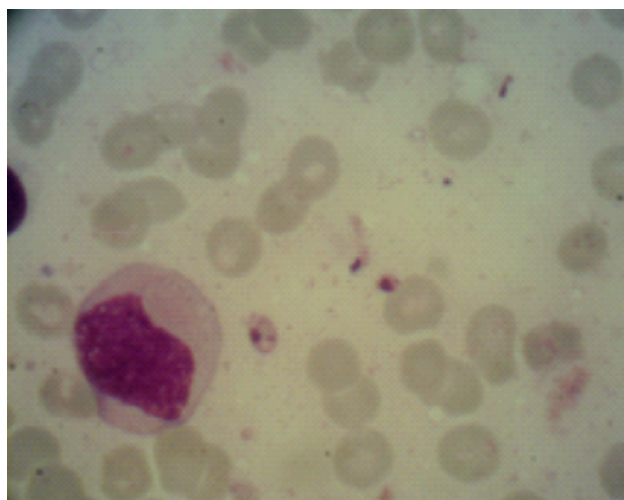


Figure 2. Leishman body (Amastigote) of *L. major*, observed in prepared samples of some patients with acute lesion, Jask county, Hormozgan province, 2008.

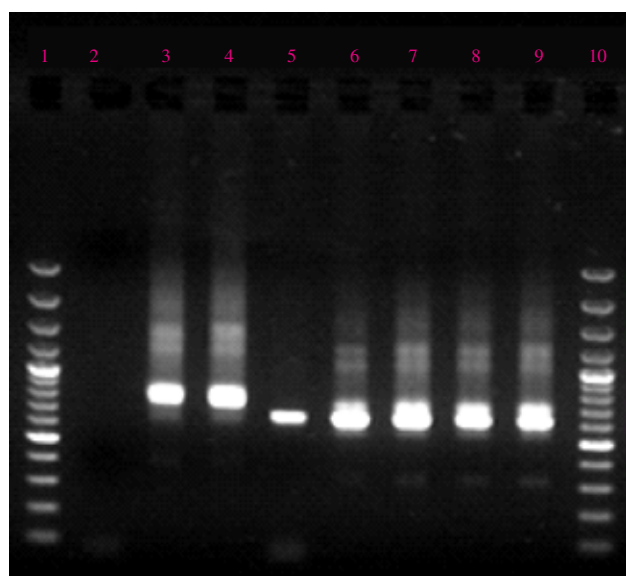


Figure 3. The results of PCR-based implication of DNA extracted from Giemsa-stained lesion smears. The bands shown, on 1.5% agarose gel stained with ethidium bromide, correspond to: Size Marker (1 & 10), Negative Control (2), *L. tropica* Standard Strain (3), *L. infantum* Standard Strain (4), *L. major* Standard Strain (5), Jask Cutaneous Leishmaniasis patient specimens (Lanes 6–9).

4. Discussion

Cutaneous leishmaniasis (CL) caused by *L. major*, *L. tropica* and visceral leishmaniasis (VL) caused by *L. infantum*, are major health problems in Iran. The majority of VL cases are reported from northwest and southwestern provinces while CL is endemic in different parts of Iran[14,16–18].

In Iran the causative agent of human cutaneous leishmaniasis have been characterized according to clinical symptoms, geographical locality, specific reservoir hosts infection, experimental animals, serology, and sometimes with species-specific monoclonal antibodies[19,20–22].

Traditionally, *Leishmania* parasites are directly detected

by microscopic examination of clinical prepared smears, however, all *Leishmania* species are very similar and their morphological species identification is so difficult[23]. Traditional diagnostic techniques (direct examination and culture) can not differentiate among *Leishmania* species and their sensitivity is low[24,25]. Although isoenzym analysis is a gold standard for differentiation of *Leishmania* species but this technique is almost expensive, time consuming and usually requires in vitro cultivation of parasite[19]. Besides, identification of the infective *Leishmania* species based on clinical signs and symptoms is not crucial because some species may cause both visceral and cutaneous involvements[4].

Several PCR-based assays targeting different genomic sources such as ssu-rRNA gene, repetitive sequences, gp63 gene locus, kinetoplast minicircle sequences and mini-exon gene sequences were developed to detect and differentiate *Leishmania* species in the vectors and reservoir hosts[8, 9, 15, 26].

In this study we used a Nested PCR and direct microscopy methods for identification of *Leishmania* species. Jask County was one of the sporadic foci of cutaneous leishmaniasis with a few cases in Eastern part of the Hormozgan province at the vicinity of Sistan–Baluchistan province. However, in recent years number of cases suddenly has increased and number of leishmaniasis cases was reported more than 240 cases in 2007. This increasing indicated a possibility of formation a major endemic focus in this part of the country. According to the topographic situation of the Hormozgan province and continuous distribution of vectors and reservoir hosts in the coastal plains, more spreading of disease to the other parts of the province was strongly suggested.

All prepared slides were checked both by microscopy and Nested-PCR. Molecular assays using specific primers showed high accuracy and more sensitivity than microscopy which is time consuming and needs master microscopists, as all of the microscopically positive slides were confirmed by Nested-PCR. So it can be concluded that nested PCR is a useful technique for studying the molecular epidemiology of leishmaniasis in the field.

Our data showed that zoonotic or rural cutaneous leishmaniasis is endemic in Jask County whose pathogen is *L. major*. In endemic areas where probably more than one *Leishmania* species are present, diagnostic tools for the detection of parasites directly in samples and distinguish all relevant *Leishmania* species are required[23].

Effective control programmes are specific for each form of disease so the species typing is completely necessary[1]. Furthermore, such information is also valuable in epidemiologic studies where the distribution of *Leishmania* species in human and animal hosts, as well as in insect vectors, is a prerequisite for designing appropriate control measures[27].

Conflict of interest statement

We declare that we have no conflict of interest.

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